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Enhanced production of specific IgG4, IgE, IgA and TGF-β in sera from patients with the juvenile form of paracoccidioidomycosis

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Paracoccidioidomycosis (PCM) occurs in two distinct forms, the acute or juvenile form (JF), and the chronic or adult form (AF). To clarify the basis of this dichotomy, specific IgG subclasses, IgA and IgE anti-gp43 were measured by enzyme-linked immunosorbent assay, in patients with different forms of PCM. Serum levels of tumor necrosis factor-α, interleukin (IL)-6, IL-8, macrophage inflammatory protein (MIP)-1α and transforming growth factor (TGF)-β were also quantified. We show here that JF patients have significantly higher titers of IgE antibodies against gp43, an immunodominant antigen specific for *Paracoccidioides brasiliensis*, than do patients with the unifocal adult form (UF-AF, isolated lesions). Patients with the multifocal adult form (MF-AF, lesions in more than one organ) also produced elevated levels of anti-*P. brasiliensis* IgE. Furthermore, specific IgE levels were correlated with IgG4, IgA and eosinophilia. Patients with JF showed eosinophilia and increased levels of TGF-β, a switching factor for IgA. These results indicate a T helper (Th)-2 pattern of cytokine expression in both the JF and the MF-AF of PCM. On the other hand, patients with UF-AF had a significantly lower production of specific IgE, IgG4 and IgA than was seen in the other patient groups.

Keywords cytokines, IgE, IgG subclasses, paracoccidioidomycosis

Introduction

Paracoccidioidomycosis (PCM), caused by the fungus *Paracoccidioides brasiliensis*, is the most prevalent deep-seated mycosis in Latin America, with a high incidence in Brazil. PCM is found mainly in former healthy individuals between 30 and 50 years old. It is uncommon in childhood and relatively rare around puberty [1,2]. A wide spectrum of clinical manifestations has been described, ranging from discrete pulmonary lesions to severe disseminated forms. According to the current classification, PCM has two distinct forms, the acute or juvenile form (JF) and the chronic or adult form (AF). The AF mainly affects adult males, who show a high frequency of pulmonary, skin and visceral involvement. In sharp contrast, the JF equally affects young patients of both sexes. The JF is characterized by systemic lymph node involvement, hepatosplenomegaly and bone marrow dysfunction, resembling a lymphoproliferative disease. From the immunopathological point of view, the JF is more frequently associated with anergic immune responses and the AF with the hyperergic responses. The JF tends to show necrotizing lesions with abundant fungal cells, impairment of cell-mediated immunity and high titers of circulating antibodies. In the AF, the lesions are seldom disseminated, and are associated with tuberculoid granulomata and a small number of fungal

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cells. Cell-mediated immunity is better preserved than in the JF and antibody levels are low [2,3].

Cellular immunity plays an essential role in controlling PCM, because the severity of the disease correlates positively with high antibody titers and impairment of T-cell function [4–7]. Presently, it is well established that resistance against the fungus is based on T-cell, macrophage and B-cell activities that are known to be mediated by interferon (IFN)-γ and other cytokines of the T helper (Th)-1 type. Conversely, susceptibility has been linked to the preferential elicitation of cytokines of the Th2 type: interleukin (IL)-4, IL-5, IL-10 and IL-13 [8]. Experimental evidence strongly suggests that, in humans, PCM resistance and susceptibility may be associated with a Th1 or a Th2 pattern of cytokine expression, respectively [9,10]. Considering that expression of immunoglobulin isotypes is determined by the patterns of cytokines present in the microenvironment where B cells are being activated [11–13], the detection of particular isotypes can be viewed as indicative of a corresponding pattern of inductive cytokines. In this study we have investigated the levels of specific anti-*P. brasiliensis* isotypes and other serum factors that could further clarify the main immunological mechanisms operating in different clinical forms of human PCM.

Material and methods

Sera

Patients cared for at the University Clinical Hospital of UNICAMP, Campinas, São Paulo, Brazil, were grouped according to the clinical form of their disease and the time spent on its treatment. The diagnosis of PCM was confirmed by histopathological exams of skin, ganglionary or pulmonary biopsy, and also by the finding of the fungus in scrapings of skin lesions, in material obtained from lymph nodes or in sputum (direct examination). We analyzed 17 sera from patients with JF (10 males and 7 females, age: 5–33) and 32 sera from patients with AF (30 males and 2 females, age: 30–72). In view of the heterogeneity of the AF of PCM, which ranges from isolated lesions in the respiratory tract to widely disseminated forms, we divided these patients into two groups: unifocal (UF-AF, 21 patients) and multifocal adult (MF-AF, 11 patients) forms. Sera were obtained before beginning, during and after treatment (serum 1, 2 and 3, respectively). Average time between collection of serum 1 and serum 2 was 11 months (10–13 months) and, between serum 2 and serum 3, 13 months (12–14 months). Twenty-three serum samples from healthy individuals with the same age range were used as control.

P brasiliensis-*gp43 antigen*

Purified gp43 was obtained by affinity chromatography of crude exoantigen of *P. brasiliensis* B-339 on Sepharose 4B (Sigma, St. Louis, MO, USA) coupled with rabbit monoclonal anti-gp43 IgG. The gp43 was eluted from this column with 0·1 M glycine–HCl, pH 2·8, immediately neutralized with 2 M Tris, pH 9·0, concentrated in an Amicon 10K apparatus (Danvers, MA, USA) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [14]. Protein content was measured by the method of Bradford [15].

Enzyme-linked immunosorbent assays (ELISA) for the determination of:

(a) IgG subclasses

Ninety-six-well plates were coated with gp43 of *P. brasiliensis*, in 0·1 M sodium carbonate buffer (pH 9·6), at 2 μg ml^{-1}, overnight at 4 °C. The plates were washed three times with phosphate-buffered saline (PBS)–0·05% Tween 20 (PBS-T). The remaining binding sites were blocked with PBS-T-5% nonfat dry milk (PBS-T-M) for 2 h at 37 °C. After being washed three times with PBS-T, 1:50-diluted serum samples were added in duplicate, and the plates were incubated for 2 h at 37 °C. The wells were washed as described above and the antibody subclasses (anti-human IgG1, IgG2, IgG3 or IgG4, Sigma) were added. After 1 h incubation at 37 °C, the plates were washed and sheep anti-mouse IgG-peroxidase (dilution 1:1000; Sigma) was added. After 1 h incubation at 37 °C and three washes with PBS-T, the substrate solution (*o*-phenylediamine in 0·1 M citrate-phosphate buffer, pH 5·0; plus 0·03% H_2O_2) was added and the reaction was interrupted by the addition of 2 N H_2SO_4. The optical densities were read in a ELISA reader (SLT Spectra, SLT Instruments, Salzburg, Austria) at 492 nm. Results were expressed as the absorbance index (AI), a numeric value calculated by dividing the net absorbance of each test serum by the net absorbance of a positive reference serum pool on each plate, multiplied by 100. The reference pool was prepared using five serum samples from patients with PCM. AI is an arbitrary value that is linearly related to the antibody concentration and allows the comparison of sera tested on different plates and in different experiments [16].

(b) Specific IgE and IgA

Ninety-six-well plates were coated with anti-IgE or anti-IgA (Sigma) in 0·1 M carbonate buffer (pH 9·6), at 2 μg ml^{-1}, overnight at 4 °C. The plates were washed three times with PBS-T and the remaining binding sites were blocked with PBS–10% fetal calf serum (PBS-FCS)

for 1 h at room temperature (RT). After plates were washed three times with PBS-T, 1:25-diluted serum samples were added in duplicate, and the plates were incubated for 2 h at 37 °C. A positive reference serum pool was included in each plate. After a further three washes with PBS-T, *P. brasiliensis* gp43 was added at 2 μg ml^{-1}, for 1 h at RT. The wells were washed three times, treated for 1 h at RT with anti-gp43 monoclonal antibody-peroxidase (dilution 1:350) and washed again. Substrate *o*-phenylediamine was added and after color development, the optical density was measured as described. The results were expressed as the corresponding absorbance index.

(c) IL-6, tumor necrosis factor (TNF)-α, IL-8 and macrophage inflammatory protein (MIP)-1α

The antibodies used for coating 96-well plates were MAB206 (anti-human IL-6), MAB210 (anti-human TNF-α), MAB208 (anti-human IL-8) and MAB670 (anti-human MIP-1α). Second-step biotinylated detection mAbs were, respectively: BAF206, BAF210, BAF208 and BAF270 (all from R&D Systems, Minneapolis, MN, USA). Concentration of cytokines in the samples was determined by a standard curve obtained with recombinant cytokines. The minimum detection limits for IL-6, TNF-α IL-8 and MIP-1α were 3·9, 7·8, 32 and 32 pg ml^{-1}, respectively.

(d) Transforming growth factor (TGF)-β

To quantify the amount of TGF-β in the serum samples, a commercial ELISA kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used. Samples were acid-activated to release all TGF-β activity by treatment with 1 N HCl (30 min), neutralized with 1 N NaOH/HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid) and diluted to 1:400. The assay was sensitive to 4 pg ml^{-1}.

Statistical methods

Patients with the JF, UF-AF and MF-AF of PCM as well as the control group were compared using the Kruskal–Wallis nonparametric test. In order to compare the variables between each phase of the treatment, for each clinical form, the ANOVA test for repeated measures was used. The relationship between parameters was examined using Spearman's rank correlation coefficients. Significance was defined as $P \leq 0{\cdot}05$.

Results

In order to evaluate IgG isotypes produced in response to *P. brasiliensis* infection, serum samples were analyzed for the detection of IgG1, IgG2, IgG3 and IgG4. As shown in Figure 1, patients with the AF of PCM (unifocal and multifocal) produced more IgG1 than patients with the JF. On the other hand, both JF and MF-AF patients produced higher levels of IgG4 anti-*P. brasiliensis* than did patients with UF-AF and the control group (Fig. 1). No significant differences were observed within to the other subclasses (IgG2 and IgG3).

Higher levels of IgE and IgA were found in JF and MF-AF patients than in UF-AF patients (Fig. 2, left and right, respectively). For IgE, no significant differences were observed between the UF-AF and control groups (Fig. 2, left).

The number of peripheral blood eosinophils was higher in patients with the JF than in those with the AF, whether UF or MF. There was a positive correlation between the levels of specific IgE and the number of peripheral blood eosinophils ($r = 0{\cdot}42$, $P = 0{\cdot}0075$, serum 1); (data not shown).

Patient follow-up showed that during treatment, IgG1 levels dropped for all patients. IgE and IgA levels also fell in both JF and MF-AF patients. The decrease in the number of peripheral blood eosinophils was very evident for patients with the JF (data not shown). For the UF-AF patients, who had presented low levels of IgG4, IgE and IgA since the beginning of infection, no differences were detected. In contrast to IgG1, IgE and IgA, the levels of specific IgG4 remained constant in both JF and MF-AF patients (Fig. 3).

The IgE antibody response to the *P. brasiliensis* gp43 antigen positively correlated with the IgG4 ($r = 0{\cdot}75$, $P < 0{\cdot}0001$) and IgA ($r = 0{\cdot}64$, $P < 0{\cdot}0001$); (data not shown).

Taking into account the prominent production of IgA in sera of both JF and MF-AF patients and based on studies showing that TGF-β is an IgA switch factor for human B cells, we next examined TGF-β levels in patients with different clinical forms of PCM. Detectable levels of TGF-β were found in all patients and in the control group (Fig. 4). JF patients produced significantly higher levels than did UF-AF ($P = 0{\cdot}04$) and MF-AF ($P = 0{\cdot}049$) patients. A positive correlation was found between IgA and TGF-β ($r = 0{\cdot}55$, $P < 0{\cdot}0001$); (data not shown).

IL-6, TNF-α and MIP-1α concentrations did not show any significant differences between the AF (UF-AF and MF-AF) and the JF. The values obtained varied widely, not allowing any correlation with the clinical forms (data not shown). In AF patients, however, significant differences were detected between the MF-AF and UF-AF, with higher levels of the inflammatory cytokines in the former [TNF-α, 136·4 vs. 4·0 pg ml^{-1}, $P = 0{\cdot}01$; IL-6, 65·1 vs. 22·3 pg ml^{-1}, $P = 0{\cdot}03$; and MIP-1α, 613·7 vs. 122·4 pg ml^{-1}, $P = 0{\cdot}03$ (data not shown)].

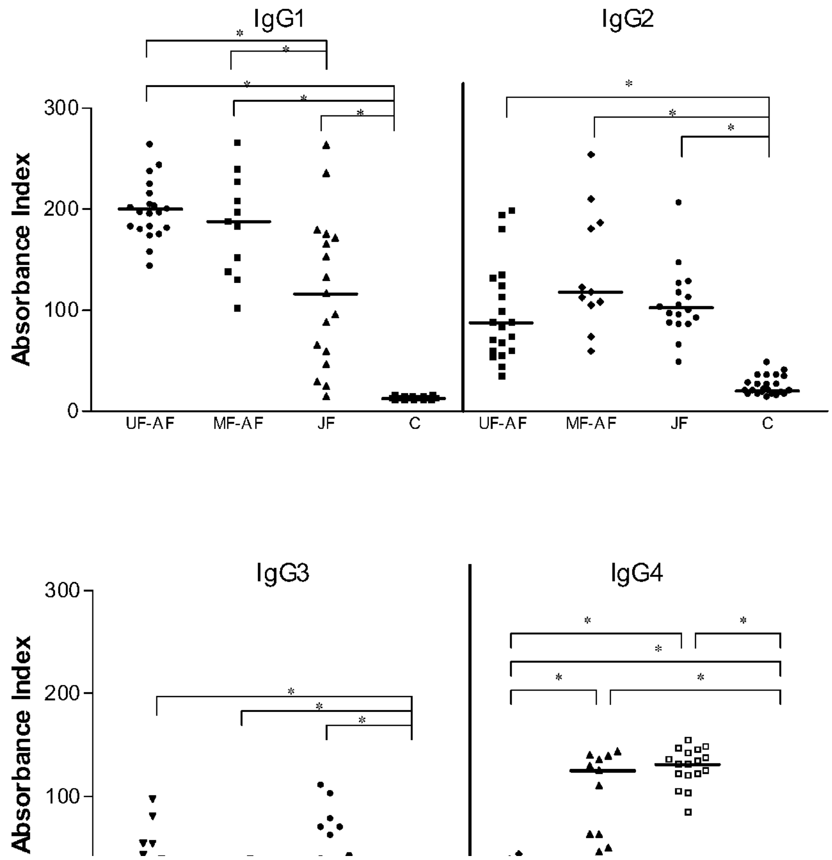

Fig. 1 Levels of anti-*P. brasiliensis* IgG1, IgG2, IgG3 and IgG4 in patients with UF-AF, MF-AF and JF PCM before treatment, and in the control group (C). The results were expressed as absorbance index. The horizontal bars represent the median. Kruskal–Wallis, $*P < 0{\cdot}05$.

As for IL-8, an alpha (CXC) chemokine described as attracting neutrophils to inflammatory sites and as inhibiting IL-4-induced IgE and IgG4 production, we found that the JF patients had lower serum levels than did UF-AF patients ($P = 0{\cdot}019$). Intermediate IL-8 levels were detected in MF-AF patients (Fig. 5).

Discussion

Th1 and Th2 subsets are defined by the production of IFN-γ, IL-2 and TNF in the former and IL-4, IL-5, IL-10, IL-13 in the latter. IFN-γ produced by Th1 cells stimulates the microbicidal activities of phagocytes, and induces the production of opsonizing and complement-fixing IgG antibodies. IL-4 and IL-13 share significant structural homology, and have many overlapping functions [11,13]. Recent data, however, strongly implicate IL-4 as the main stimulus for the induction of Th2 cells and IgE production, whereas IL-13 seems to be more efficient in later Th2, including eosinophilic inflammation [17]. IL-5, in addition to activating eosinophils,

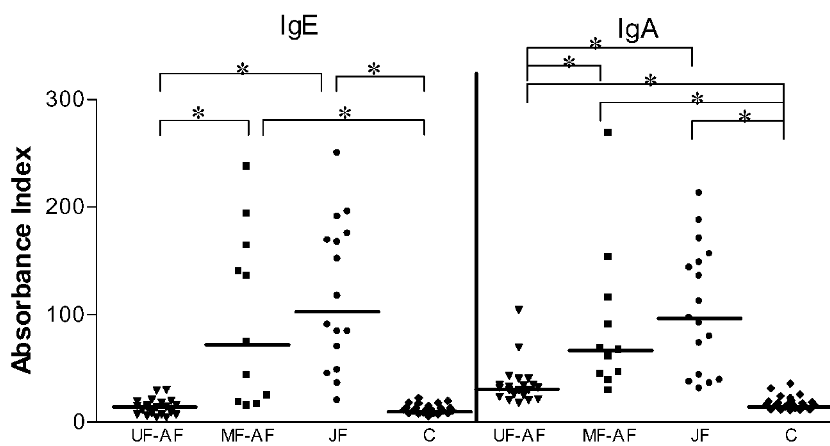

Fig. 2 Levels of anti-*P. brasiliensis* IgE (left) and IgA (right) in sera of patients with UF-AF, MF-AF and JF PCM, before treatment, and in the control group (C). The results were expressed as absorbance index. The horizontal bars represent the median. Kruskal–Wallis, $*P < 0{\cdot}05$.

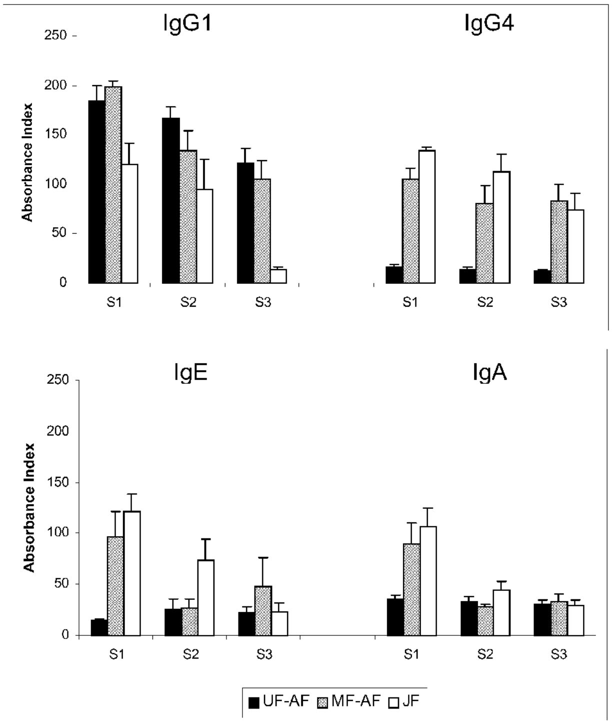

Fig. 3 Levels of anti-*P. brasiliensis* IgG1, IgG4, IgE and IgA in consecutive sera from patients with the UF-AF, MF-AF and the JF PCM. Sera were obtained before the beginning (S1), during (S2) and after treatment (S3). The average time between the collection of sera was 12 months. Significant differences between consecutive sera from the same patient: IgG1:UF-AF, MF-AF and JF–S1 versus S2, S2 versus S3, S1 versus S3; IgE:MF–S1 versus S2, JF–S1 versus S2, S2 versus S3, S1 versus S3; IgA:MF–S1 versus S2, S1 versus S3, JF–S1 versus S2, S1 versus S3. The results were expressed as absorbance index (mean ± SEM). ANOVA for repeated measures, $P < 0.05$.

stimulates the production of IgA, in synergism with TGF-β [12,18,19].

The excellent correlation between IL-4 and IgE suggests that IgE production indicates a strong Th2-like response [11,13]. Other studies have shown that high levels of polyclonal IgE are associated with impairment of T-cell-mediated immunity [20,21]. Using a technique we developed [22], we were able to show that both JF and MF-AF patients have high titers of IgE antibodies to gp43, an immunodominant antigen specific for *P. brasiliensis* [23]. Gp43 is a glycoprotein of 43 000 Da that can be isolated from the supernatant fluid of yeast cultures by affinity chromatography. This molecule specifically binds to the extracellular matrix protein laminin, facilitating pathogenesis [24,25]. Patient follow-up showed that although IgE antibodies consistently dropped in treated patients, IgG4 levels tended to remain stable. Occurrence of specific IgG4 antibodies in PCM was recently described by Baida *et al.* [26]. These authors found anti-gp43 IgG4 antibodies in sera of 100% of the patients with the JF, and in only 12% of those with the AF of PCM. We were able to show in the present study that MF-AF patients also produce high levels of anti-*P. brasiliensis* IgG4 and IgE antibodies, as well as low levels of IgG1. Therefore, we conclude that IgG4 and IgE may be useful markers of disease severity and of impairment of the protective immunity.

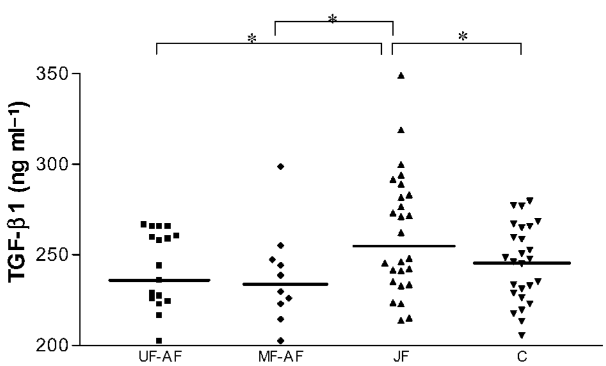

Fig. 4 Levels of TGF-β in sera from patients with UF-AF, MF-AF, JF PCM and the control group (C). Kruskal–Wallis, $*P < 0.05$. The horizontal bars represent the median.

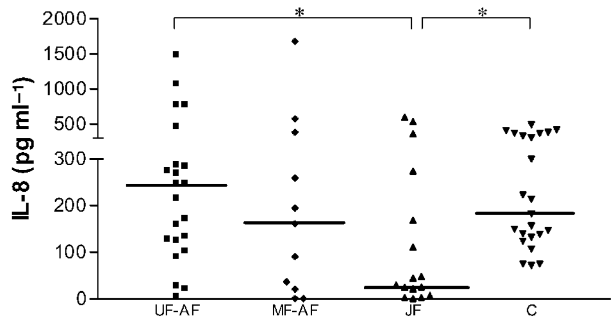

Fig. 5 Levels of IL-8 in sera from patients with UF-AF, MF-AF, JF PCM and the control group (C). Kruskal–Wallis, $*P < 0.05$. The horizontal bars represent the median.

IgG4 antibodies against some antigens are functionally monovalent and give rise to small nonprecipitating immune complexes that do not fix complement [27] and, therefore, have a low potential for destroying pathogens. All the signals recognized to be involved in IgE switching *in vitro* also control IgG4, suggesting that IgE and IgG4 synthesis are regulated by common mechanisms [13,28]. However, many observations indicate that *in vivo* these isotypes may be independently regulated [29,30]. In chronic helminthic infections, IgG4 antibodies are the predominant IgG subclass and probably play an important role as 'blocking antibodies' of IgE mediated response. Furthermore, the development of a potent and specific IgG4 response, during allergic desensitization, has been associated with a positive outcome [28,31]. From these observations, we

hypothesize that specific IgG4 may have a regulatory role in IgE mediated response in PCM.

The most interesting observation of our study was the strong positive correlation between specific IgE levels and IgG4, IgA and eosinophilia. We observed that JF patients had significantly higher numbers and percentages of peripheral eosinophils than did AF patients. The degree of eosinophilia was positively correlated with IgE levels and both parameters decreased with treatment. These results agree with those of Benard *et al*. [6], who showed an inverse correlation between eosinophil levels and T cell function, evaluated by the *in vitro* proliferative response to *P. brasiliensis* antigens. In coccidioidomycosis, peripheral blood eosinophilia and microabscesses with large numbers of eosinophils were related to the disseminated form of the disease and to a poor prognosis [32]. A recent paper suggested that eosinophils, through toxic granule proteins, could participate in PCM pathophysiology [33]. These observations could be taken as evidence for IL-5 production, because the number of eosinophils in the circulation is directly influenced by this cytokine [34].

In experimental PCM, a preferential secretion of specific IgA was associated with progressive disease in susceptible mice [35]. However, the data concerning IgA antibody production in both forms of human PCM are scarce and controversial, with only two papers describing opposite results [25,36]. In the present study, we detected higher IgA-specific antibody levels in the JF and in MF-AF than in UF-AF patients. Moreover, we also detected increased levels of TGF-β in the sera of JF patients. TGF-β is the cytokine responsible for IgA switch [18], in addition to a variety of other immunological effects such as inhibition of the proliferative response of T cells and impairment of macrophage activation [19]. In leishmaniasis, endogenous TGF-β production correlates with susceptibility to infection and with the development of a nonhealing Th2-type response [37].

Silva *et al*. [38] suggested a role for inflammatory cytokines in the genesis and control of PCM. These authors observed increased levels of TNF, IL-1 and IL-6 in serum of adult PCM patients with disseminated disease. These findings were associated with low lymphoproliferative response and high antibody titers. In the present study, the evaluation of IL-6, TNF-α and MIP-1α concentrations did not distinguish the JF from the AF, either before treatment or after it. However, high levels of these cytokines were detected in MF-AF as compared with UF-AF patients. IL-8 levels in patients with the JF were significantly lower than those seen in UF-AF patients. This may be an additional indicator of a predominantly Th2 type response in JF. IL-8 modulates the IgE synthesis induced by IL-4 [39] and, therefore, a reduction in the production of IL-8 could contribute to a shift toward a Th2 response in the JF. Furthermore, as IL-8 is a potent chemokine for neutrophils, mobilization of these cells to the inflammatory sites could be altered in the JF patients, contributing to disease dissemination. A complementary paper in which we evaluate the lymphoproliferative response and Th1 vs. Th2 cytokines production in patients with different clinical forms of PCM is in preparation.

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